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(74) **Mandataire** :

(54) MILIEU DE CULTURE POUR TISSUS MERISTEMATIQUES ET PROCEDE DE CULTURE DE CES TISSUS EN CONDITION NON STERILES.

(57) Milieu de culture gelosé contenant du sucre pour tissus méristématiques.

Il contient en outre une quantité efficace et non phytotoxique d'au moins une matière active protectrice contre les micro-organismes choisis dans le groupe comprenant une matière active fongicide phytosanitaire et une matière active bactéricide rémanente.

Application en agriculture.

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Culture medium for meristematic tissues and a process for the culture of such tissues in non-sterile conditions.

The invention refers to a culture medium for meristematic tissues, a process for the culture of such tissues in non-sterile conditions, and the plants obtained by means of that process.

It is known to art that the culture of somatic embryos of plants and of seedlings grown from them requires the use of a sugar culture medium. However, the presence of sugar attracts micro-organisms like fungi, bacteria, algae, protozoa and viruses. And yet a maximum protection is required to ensure the culture, growth and obtaining of seedlings and thus of health plants. This drawback is avoided by carrying out the culture in sterile conditions, which burdens and complicates experimentation. Another method is to add antibiotics. This solution has been tried, for instance, in the culture of carrot embryos, cf. Synseeds 1993 Ch; 15 F. Molle et al., but was found to be unusable owing to an unacceptable phytotoxicity which blocked embryo growth. Wide-spectrum fungicides such as carbendazime or benomul have been used, but they have only been shown to be non-phytotoxic at weak doses, and thus their action was entirely insufficient. The same holds true for certain combinations of fungicides and antibiotics found in the literature.

It is therefore known that the problem of effective protection of culture media for somatic embryos in non-sterile conditions remains unsolved and that there is a need to resolve this difficulty. This difficulty results from the greater sensitivity, in non-sterile conditions, of embryos as opposed to seeds, and of embryos to phytosanitary products, as well as to the fact that the embryos must also be protected against the actions of micro-organisms other than pathogens.

The applicant has now discovered a new culture medium allowing this problem to be resolved by obtaining effective protection through the use of special active protective ingredients. By effective protection is meant a protection of at least 10% of the surface of the medium.

More particularly, the invention concerns a culture medium for meristematic tissues, in non-sterile conditions, comprising a sugar medium, characterized in that it contains additionally an effective and non-phytotoxic quantity of at least one active protective ingredient against micro-organisms selected in the ground including a phytosanitary active fungicidal ingredient and a persistent active bactericidal ingredient.

In the sense of the present description, "meristematic tissue" means any plant tissue, or assembly of plant cells, capable, when it is placed in suitable conditions, of developing until it forms a whole plant or a part of a whole plant. Under the expression "tissue" is included every kind of plant tissue, and more particularly somatic tissue, pre-embryogenic masses, somatic embryos, zygotic tissue, seed, germ, adventitious buds, sprouts, shoot primordium, the tissue known in English as the green spot, germ cells and germ lines, and young plants.

The plants concerned by the invention are by nature extremely diverse and include food crops such as rice, wheat, barley, corn, and soybeans; vegetable crops such as celery, parsley, lettuce, cauliflower, carrots, eggplants, tomatoes, onions, garlic, ginger, strawberries, melons, and asparagus; food and/or cash crops like rape, sugarcane, sugar beets, tobacco, coffee; crops for medicinal purposes such as belladonna, ginseng; ornamental crops such as chrysanthemums, gladioli, lilies, orchids, amaryllis, geraniums, begonias, African violets, poinsettia; trees or saplings or shrubs such as conifers, palms, fruit trees, grape vines, deciduous trees, and others.

The meristematic tissue may be dry, dehydrated or in a hydrated form with up to 100% humidity.

According to the present invention, by "micro-organisms" is understood those belonging to the classes of fungi, bacteria, algae, virus and protozoa.

Similarly, by "culture medium" is understood any medium, solid (for example, agar medium) or liquid (nutrient solution) that may be absorbed by culture substrates

such as rock wool, cellulose acetate, polyethylene or polyurethane fibers, or coconut, and which permit the culture of meristematic tissues, *in vitro* or in a greenhouse, and as necessary with controlled release of the active ingredients. The medium may be static, applied one time only, or renewed regularly in the form of either a continuous flow or a discontinuous flow (batch).

The sugar is present in the medium according to the invention at a concentration generally ranging between 0.1 mg and 200 g/l, and preferably between 5 g/l and 200 g/l.

By "active phytosanitary ingredient" is understood essentially an active fungicidal ingredient. This is preferably selected from a group including derivatives of copper, derivatives of oxyquinolein, dithiocarbonates, dicarboximides, phenylpyrroles, or triazoles. In an especially preferred embodiment, fungicides are chosen from a group including copper oxychloride, mancozeb, maneb, thiram, fludioxonil and iprodione.

In the present application, the names of the fungicidal ingredients used are the common names (cf. *Pesticide Manual* 1995).

In a particularly preferred embodiment, the medium contains at least two fungicides.

Another important aspect of the invention is that the fungicides are used in quantities that are not phytotoxic for the meristematic tissue, that is in concentrations that may be weaker than those customarily used for the protection of whole plants. More specifically, the medium contains from 0.1 mg to 10 g/l, and preferably from 1 mg to 1 g/l of active fungicidal ingredient.

More particularly, and preferably, the concentration ranges between:

- 0.1 and 10 mg/l for copper derivatives;
- 0.1 and 10 mg/l for derivatives of oxyquinolein;
- 0.1 and 10 mg/l for dithiocarbamic derivatives;

- 0.1 and 10 mg/l for phenylpyrrole derivatives;
- 0.1 and 1000 mg/l for dicarboximide derivatives.

By "active bactericidal ingredient" is meant essentially a bactericidal or bacteriostatic active ingredient. This may belong to any family, preference being accorded to derivatives of salicylic acid such as salicylic acid, acetylsalicylic acid, and salicylates or as well to thiazolines, such as 1,2-benzisothiazolin-3-one, or antibiotics.

The medium may contain a number of bactericides.

The concentration of active bactericidal ingredient is from 0.1 to 100 mg/l, and preferably from 0.1 to 100 and preferably from 0.1 to 10 mg./l of active ingredient.

More particularly and preferably, the concentration is between:
- 0.1 and 10 mg/l for derivatives of salicylic acid;
- 0.1 and 5 mg/l for 1,2-benzisothiazolin-3-one.

The medium according to the invention may as well contain other active ingredients or adjuvants provided that the quantities are not phytotoxic for the meristematic tissues. For the preparation of the medium itself, it is sufficient to mix the ingredients described with the base of the medium. If the final medium is solid, the mixture is performed hot in the liquid phase for subsequent pouring into laboratory recipients such as Petri dishes or Magenta vessels.

Culture conditions and results will be better understood with the aid of the following examples, which in no way limit the scope of the invention.

<u>Example 1: Culture medium protection test</u>

Different culture media are prepared starting with the Heller nutrient solution (He 15), characterized by the presence of sucrose, which is used to ensure the germination of the somatic embryos. This solution is composed of Heller macronutrients (***Ann. Sci. Nat.***

Bot. Biol. Veg. 14:1-223, 1953), the Murashige and Skoog micronutrients (*Physiol. Plant.* 15: 473-497, 1962), and 15 g/l of sucrose. In the example, it is gelled with 6 g/l of Gelrite, pH being maintained at 5.6. After preparation and sterilization by autoclaving about 3.5 liters of Heller gelled nutrient solution (He 15), about 250 ml of the following Heller media are prepared.

1. Heller without active ingredient.
2. Heller + oxyquinolein 1 mg/l + thiram 5 mg/l
3. Heller + mancozeb 10 mg/l
4. Heller + copper oxyquinoleate 1 mg/l + mancozeb 10 mg/l
5. Heller + copper oxyquinoleate 1 mg/l + fludioxonil 10 mg/l
6. Heller + copper oxyquinoleate 1 mg.; + iprodione 100 mg/l.

The modalities of preparation and dilution of the stock solutions of the active ingredients (a.i.) are given in Table 1 below:

Active ingredient	Formulation	Quantity of a.i per liter of stock solution	Volume of stock solution per liter of culture medium
oxyquinolein	cryptonol	0.2	5
copper oxyquinoleate	technical a.i. 100%	1	1
thiram	Pomarsol	1	5
mancozeb	Dithane M45	2	5
fludioxonil	Saphire	2	5
iprodione	Rovral aqua flo	2	5

The stock solution is prepared by dilution of the active ingredient, prepared or not, in a sucrose-free Heller solution. The culture medium is prepared by adding a fraction of the stock solution to the base medium, which contains 15 g/l of sucrose.

Each gelled nutrient solution is distributed into Magenta vessels (Sigma, U.S.A.) at approximately 40 ml per vessel. The Magenta vessels are housed in mini-greenhouses which are opened without any special precautions in non-sterile conditions, one vessel per medium and per mini-greenhouse.

The temperature is about 20°C, with relative humidity adjustable from 30 o 70% by means of a fogging system. The greenhouses allow the use of natural light, but if that is too weak, fixed supplementary lighting is used in the form of 400-Watt high-pressure sodium vapor lamps.

Four days after placing them, the surface of the culture medium contaminated by micro-organisms and the number of colonies developed are measured in each vessel.

Results are given in Table 2 below:

Active ingredients	Number of colonies	Contaminated surface		
		mm^2	% of total surface	% of control surface
0	108	17906	86	100
mancozeb 10 mg/l	1	104	0.5	0.6
oxquinolein 1 mg/l + thiram 5 mg/l	5	766	4	4
copper oxyquinoleate 1 mg/l + mancozeb 10 mg/l	0	38	0	0
copper oxyquinoleate 1 mg/l + iprodione 100 mg/l	38	1763	8	10

The criterion of activity is that the protection be such that less than 10% of the total surface is contaminated.

Under these conditions we observe that all the products tested provide excellent protection.

Example 2: Culture medium protection test

Proceeding under the same protocol as that described for Example 1, but with the following Heller media:

7. Heller without active ingredient
8. Heller + copper oxyquinoleate 10 mg/l
9. Heller + copper oxyquinoleate 1 mg/l + thiram 5 mg/l,

we obtain the results given in Table 3 below:

Active ingredients	Number of colonies	Contaminated surface		
		mm²	% of total surface	% of control surface
0	18	4574	22	100
copper oxyquinoleate 10 mg/l	1	3	0.0	0.0
copper oxyquinoleate 1 mg/l + thiram 5 mg/l	3	129	0.6	3

Example 3: Culture medium protection test

Proceeding under the same protocol as that described for Example 1, but with the following Heller media:

10. Heller without active ingredient
11. Heller + copper oxyquinoleate 1 mg/l
12. Heller + thiram 10 mg/l
13. Heller + salicylic acid 10 mg/l
14. Heller + 1,2-benzisothiazolin-3-one 10 mg/l
15. Heller + copper oxyquinoleate 1 mg/l + thiram 5 mg/l
16. Heller + copper oxyquinoleate 1 mg/l + thiram 5 mg/l + salicylic acid 1 mg/l

17. Heller + thiram 5 mg/l + salicylic acid 1 mg/l

18. Heller + thiram 5 mg/l + 1,2-benzisothiazolin-3-one 10 mg/l.

These gelled culture media are prepared from the Heller nutrient solution (He 15), whose composition is described in the table for Example No. 1.

Modalities of preparation and dilution were given in the preceding examples. In the case of salicylic acid and 1,2-benzisothiazolin-3-one, these modalities are given in Table 4 below:

Active ingredient in g of a.i. per liter of culture medium	Formulation	Quantity in g of a.i. per liter of stock solution	Volume in ml of stock solution per liter of culture medium
salicylic acid: 10 g/l 1 g/l	Sygma ref. no. S 3007, U.S.A.	10 1	1
1,2-benzisothiazoline-3-one: 10 g/l	20% liquid formulation	1	10

The results shown in Table 5 below are obtained:

Active ingredients	Number of colonies	Contaminated surface		
		mm²	% of total surface	% of control surface
0	92	19249	92	100
copper oxyquinoleate 1 mg/l	35	5939	26	28
thiram 10 mg/l	2	69	0.3	0.3
salicylic acid 10 mg/l	90	18518	89	96
1-2-benzisothiazolin-3-one 10 mg/l	7	1042	5	5

copper oxyquinoleate 1 mg/l + thiram 5 mg/l	6	1635	8	8
copper oxyquinoleate 1 mg/l + thiram 5 mg/l + salicylic acid 1 mg/l	5	590	3	3
thiram 5 mg/l + salicylic acid 1 mg/l	4	1392	7	7
thiram 5 mg/l + 1,2-benzisothiazolin-3-one 10 mg/l	2	869	4	5

Under these conditions we observe that thiram, benzisothiazolin-3-one and mixtures based on copper oxyquinoleate and acetylsalicylic and/or thiram, at doses where these products by themselves are ineffective, provide an excellent protection, such that less than 10% of the total surface is contaminated.

Example 4: Culture test with carrot somatic embryos

A) Cellular suspension:

The somatic embryos are obtained according to the method described in *Cryo-Lett.* 12: 319-328, 1991), or else embryogenesis is obtained in the presence of 0.2%° by weight of activated carbon, and they are dehydrated according to the method described in *C.R. Acad. Sci. Paris*, Ser. III, 314, 423, 1992. The embryos thus obtained have a water content of less than 0.35 grams of water per gram of dry material.

B) Germination of embryos:

The Petri dishes containing the embryos laid on filter paper are placed in darkness for 3 days at 4°C in dessicators where the relative humidity is maintained at 96% by a supersaturated solution of potassium nitrate. Next, the filter papers are transferred in darkness and at 4°C onto a Heller medium at 145 g/l of sucrose. At the end of one day, they are again moved onto a Heller medium at 80 g/l of sucrose, under light, and at 25°C. At the end of one day, these embryos are ready for use.

The somatic embryos are placed in germination in a growth chamber at 25°C under a photoperiod of 15 h of day and 8 h of night and a light intensity of 15 µE/m²/s. The germination medium is a gelled Heller medium containing 15 g/l of sucrose, with or without active ingredients added. Somatic embryos are assigned to different media on the basis of 60 embryos per medium and 20 embryos per vessel, under aseptic conditions.

The various Heller media containing the mixtures of active ingredients are prepared as in the preceding examples:

19. Heller without active ingredient
11. Heller + copper oxyquinoleate 1 mg/l + mancozeb 10 mg/l
5. Heller + copper oxyquinoleate 1 mg/l + fludioxonil 10 mg/l
6. Heller + copper oxyquinoleate 1 mg/l + iprodione 100 mg/l
20. Heller + copper oxyquinoleate 1 mg/l + thiram 5 mg/l
16. Heller + copper oxyquinoleate 1 mg/l + thiram 5 mg/l + salicylic acid 1g/l*
18. Heller + thiram 5 mg/l + 1,2-benzisothiazolin-3-one 1 mg/l
21. Heller + copper oxyquinoleate 1 mg/l + fenpiclonil 1 m/gl [*sic*].

The rates of conversion of the embryos are measured about 5 weeks after seedling and defined as the number of embryos showing the first pair of real leaves in relation to the total number of embryos.

The wet weight of the stem of the young plant (above the root crown) is measured on an analytic balance. The wet weight is the market for plant development.

Conversion rates and wet weights are shown in Table 6 below.

* *Translator's note:* This formulation earlier showed salicylic acid 1 mg/l.

Active ingredients	Conversion rate (%)	Mean wet weight of plants (mg)
none	95	117 ± 8
copper oxyquinoleate 1 mg/l + mancozeb 10 mg	83	124 ± 15
copper oxyquinoleate 1 mg/l + fludioxonil 10 mg/l	95	115 ± 12
copper oxyquinoleate 1 mg/l + thiram 5 mg/l	95	115 ± 12
copper oxyquinoleate 1 mg/l + iprodione 100 mg/l	98	126 ± 3
copper oxyquinoleate 1 mg/l + thiram 5 mg/l + salicylic acid 1 mg/l	90	104 ± 17
thiram 5 mg/l + 1,2-benzisothiazolin-3-one 1 mg/l	98	142 ± 10
copper oxyquinoleate 1 mg/l + fenpiclonil 1 m/gl [*sic*]	95	114 ± 15

This table shows that all the rates of conversion into plants are superior to the acceptable value of 80%, and even for most of them at least equal to 95%. This demonstrates the excellent property of the active ingredients according to the invention of not altering the development of somatic embryos into plants.

Claims

1. Culture medium for meristematic tissues, in non-sterile conditions, comprising a sweetened medium, and characterized in that it additionally contains an effective and non-phytotoxic quantity of at least one active ingredient protective against micro-organisms, selected from the group containing a phytosanitary fungicidal active ingredient and a persistent bactericidal active ingredient.

2. Medium according to claim 1, characterized in that the active protective ingredient is a fungicidal active ingredient.

3. Medium according to claim 2, characterized in that the active fungicidal ingredient is a copper derivative.

4. Medium according to claim 3, characterized in that the active fungicidal ingredient is a derivative of copper oxychloride.

5. Medium according to claim 2, characterized in that the active fungicidal ingredient is a derivative of oxyquinolein.

6. Medium according to claim 5, characterized in that the active fungicidal ingredient is copper oxyquinoleate.

7. Medium according to claim 2, characterized in that the active fungicidal ingredient is a dithiocarbamate.

8. Medium according to claim 7, characterized in that the active fungicidal ingredient is mancozeb.

9. Medium according to claim 7, characterized in that the active fungicidal ingredient is thiram.

10. Medium according to claim 2, characterized in that the active fungicidal ingredient is a phenylpyrrole derivative.

11. Medium according to claim 10, characterized in that the active fungicidal ingredient is fludioxonil.

12. Medium according to claim 2, characterized in that the active fungicidal ingredient is a dicarboximide.

13. Medium according to claim 12, characterized in that the active fungicidal ingredient is iprodione.

14. Medium according to one of claims 2 to 13, characterized in that the medium contains at least two fungicides.

15. Medium according to claim 14, characterized in that one of the two fungicides is a derivative of oxyquinolein or a dithiocarbamate.

16. Medium according to one of claims 2 to 15, characterized in that the medium contains from 0.1 to 1000 mg/l, and preferably from 0.1 to 10 mg./l, of active fungicidal ingredient.

17. Medium according to claim 1, characterized in that the active protective ingredient is a persistent bactericidal active ingredient.

18. Medium according to claim 17, characterized in that the active bactericidal ingredient is a salicylate.

19. Medium according to claim 17, characterized in that the active bactericidal ingredient is 1,2-benzisothiazolin.

20. Medium according to one of claims 17 to 19, characterized in that the medium contains from 0.1 to 100 mg/l, and preferably from 0.1 to 10 mg/l of active bactericidal ingredient.

21. Medium according to one of claims 1 to 20, characterized in that the medium contains at least one active fungicidal ingredient.

22. Medium according to claim 1, characterized in that the sugar concentration is at least equal to 1 g/l.

23. Medium according to one of claims 1 to 22, characterized in that it includes a fibrous phase for the controlled release of the active ingredients.

24. Process for growing a meristematic tissue in non-sterile conditions, characterized in that it uses a medium according to one of the claims 1 to 23.